

Date: May 21, 1998

**TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 USC 371**

International Application No.: PCT/BE96/00123
International Filing Date: November 21, 1996
Priority Date Claimed: November 21, 1995
Title of Invention: RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING SAID
RECEPTOR
Applicant(s) for DO/EO/US: Communi, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.
2. () This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371.
3. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).
4. (X) A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. (X) A copy of the International Application as filed (35 USC 371(c)(2))
 - a. () is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. (X) has been transmitted by the International Bureau.
 - c. () is not required, as the application was filed in the United States Receiving Office (RO/US).
6. () A translation of the International Application into English (35 USC 371(c)(2)).
7. (X) Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
 - a. () are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. (X) have been transmitted by the International Bureau.
 - c. () have not been made; however, the time limit for making such amendments has NOT expired.
 - d. () have not been made and will not be made.
8. () A translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).
9. () An oath or declaration of the inventor(s) (35 USC 371(c)(4)).
10. (X) A copy of the International Preliminary Examination Report with any annexes thereto, such as any amendments made under PCT Article 34.
11. () A translation of the annexes, such as any amendments made under PCT Article 34, to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

12. () An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
13. () An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

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Date: May 21, 1998

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25. (X) The fee for later submission of the signed oath or declaration set forth in 37 CFR 1.492(e) will be paid upon submission of the declaration.
26. (X) A check in the amount of \$652 to cover the above fees is enclosed.
27. () Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40 per property.
28. (X) The Commissioner is hereby authorized to charge only those additional fees which may be required to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Applicant or Patentee: Communi et al.

Application or Patent No.: 09/077,173

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For: **RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING SAID RECEPTOR**

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VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL-ENTITY STATUS

I, the undersigned, do hereby declare that:

☒ I am an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: EUROSCREEN S.A.

ADDRESS OF CONCERN: Avenue des Becassines 7, B-1160 Bruxelles, Belgium

I further declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both. I further declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in the patent or application identified above.

The individual, concern or organization identified above has not assigned, granted, conveyed or licensed, and is under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

If the rights held by the above-identified individual, concern or organization are not exclusive, each individual, concern or organization having rights in the invention are identified below. Each such individual, concern or organization must file separate verified statements averring to their status as small entities.

***NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).**

FULL NAME:

ADDRESS:

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small-entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: *Pierre Nokin*

TITLE OF PERSON (if not an owner or individual): *Managing Director*

ADDRESS OF PERSON SIGNING: EUROSCREEN S.A., Avenue des Becassines 7, B-1160, Bruxelles, Belgium

SIGNATURE: _____

NSB-5653 cd/060998

DATE: September 2, 1998

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10 RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING SAID RECEPTOR.Object of the present invention.

15 The present invention concerns a new receptor having a preference for pyrimidine nucleotides preferably uridine triphosphate over purine nucleotides and the nucleic acid molecule encoding said receptor, vectors comprising said nucleic acid molecule, cells transformed by said vector, antibodies directed against said receptor, nucleic acid probes directed against said nucleic acid molecule, 20 pharmaceutical compositions comprising said products and non human transgenic animals expressing the receptor according to the invention or the nucleic acid molecule according to said receptor.

25 The invention further provides methods for determining ligand binding, detecting expression, screening for drugs, molecular binding specifically to said receptor and treatment involving the receptor according to the invention.

Background of the invention.

30 The cloning of several receptors for ATP has been reported since 1993. In keeping with the latest nomenclature proposal, these P2 purinergic receptors can be subdivided

into two classes: G protein-coupled receptors, or P2Y receptors, and receptors with intrinsic ion channel activity or P2X receptors (2). Two distinct rat P2X receptors have been cloned, respectively from the vas deferens (3) and phaeochromocytoma PC12 cells (4): they have a characteristic topology, with two hydrophobic putatively membrane-spanning segments and an ion pore motif reminiscent of potassium channels. In the P2Y family, the sequences of two subtypes, both coupled to phospholipase C, have been published: chick (5), turkey (6), bovine (7), mouse and rat (8) P2Y1 receptors (formerly called P2Y); murine (9,10), rat (11) and human (12) P2Y2 receptors (previously named P2U) on the other hand. In addition, a P2Y3 receptor, with a preference for ADP over ATP, has been cloned from chick brain, but its sequence is not yet published (13). Furthermore, the 6H1 orphan receptor, cloned from activated chicken T lymphocytes, exhibits a significant degree of homology to the P2Y1 and P2Y2 receptors, suggesting that it also belongs to the P2Y family, although its responsiveness to nucleotides has not yet been demonstrated (14).

Summary of the invention.

This invention provides a receptor having a preference for pyrimidine nucleotides preferably uridine triphosphate over purine nucleotides. A receptor having a preference for pyrimidine nucleotides over purine nucleotides means a receptor for which pyrimidine nucleotides and purine nucleotides are not equally active and equipotent. This means that the receptor according to the invention in presence of these agonists presents a functional response (preferably the accumulation of Inositol triphosphate (IP3), diacylglycerol (DAG), or calcium ions) to lower concentration of pyrimidine nucleotides, preferably uridine triphosphate, than to purine

nucleotides or a more important functional response to similar concentration of pyrimidine nucleotide than to purine nucleotide.

The inositol phosphate (IP3) accumulation after addition of said agonists is described in the specification thereafter.

Advantageously, the receptor according to the invention has at least a twofold, preferably a tenfold to one hundredfold preference for pyrimidine nucleotides over purine nucleotides.

A preferred embodiment of the receptor according to the invention is characterized by a preference for uridine triphosphate over adenine nucleotides.

The receptor according to the invention is a receptor, preferably a G protein-coupled receptor, which belongs structurally to the purinergic receptor family (P2Y family) but functionally is a pyrimidinergic receptor, preferably a UTP-specific receptor.

According to a preferred embodiment of the present invention, the receptor is a human receptor.

Said receptor has an amino acid sequence having more than 60% homology with the amino acid sequence shown in figure 1. Preferably, the amino acid sequence of the receptor according to the invention has at least the amino acid sequence shown in figure 1 or a portion thereof.

A portion of the amino acid sequence means a peptide or a protein having the same binding properties as the receptor according to the invention (i.e. peptide or a protein which is characterized by a preference for pyrimidine nucleotides, preferably UTP, over purine nucleotides).

The present invention is also related to a nucleic acid molecule, such as a DNA molecule or an RNA molecule,

encoding the receptor according to the invention.

Preferably, said DNA molecule is a cDNA molecule or a genomic DNA molecule.

5 Preferably, said nucleic acid molecule has more than 60% homology to the DNA sequence shown in figure 1.

10 Preferably, the nucleic acid molecule according to the invention is at least the DNA sequence shown in figure 1 or portion thereof. "A portion of a nucleic acid sequence" means a nucleic acid sequence encoding at least a portion of amino acid sequence as described above.

15 The present invention is also related to a vector comprising the nucleic acid molecule according to the invention. Preferably, said vector is adapted for expression in a cell and comprises the regulatory elements necessary for expressing the amino acid molecule in said cell operatively linked to the nucleic acid sequence according to the invention as to permit expression thereof.

20 Preferably, said cell is chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells. The vector according to the invention is a plasmid or a virus, preferably a baculovirus, an adenovirus or a semliki forest virus.

The plasmid may be the pcDNA3-P2Y4.

25 The present invention concerns also the cell (preferably a mammalian cell, such as a 1321N1 cell) transformed by the vector according to the invention. Advantageously, said cell is preferably non neuronal in origin and is chosen among the group consisting of a COS-7 cell, an LM(tk-) cell, an NIH-3T3 cell or a 1321N1 cell.

30 The present invention is also related to a nucleic acid probe comprising the nucleic acid molecule according to the invention, of at least 15 nucleotides capable of

specifically hybridizing with a unique sequence included in the sequence of the nucleic acid molecule encoding the receptor according to the invention. Said nucleic acid probe may be a DNA or an RNA molecule.

5 The invention concerns also an antisense oligonucleotide having a sequence capable of specifically hybridizing to an mRNA molecule encoding the receptor according to the invention so as to prevent translation of said mRNA molecule or an antisense oligonucleotide having a
10 sequence capable of specifically hybridizing to the cDNA molecule encoding the receptor according to the invention.

Said antisense oligonucleotide may comprise chemical analogs of nucleotide or substances which inactivate mRNA, or be included in an RNA molecule endowed with ribozyme
15 activity.

Another aspect of the present invention concerns a ligand other than purine and pyrimidine nucleotides (preferably an antibody) capable of binding to a receptor according to the invention and an anti-ligand (preferably
20 also an antibody) capable of competitively inhibiting the binding of said ligand to the receptor according to the invention.

Preferably, said antibody is a monoclonal antibody.

The present invention concerns also the monoclonal
25 antibody directed to an epitope of the receptor according to the invention and present on the surface of a cell expressing said receptor.

The invention concerns also the pharmaceutical composition comprising an effective amount of oligonucleotide
30 according to the invention, effective to decrease the activity of said receptor by passing through a cell membrane and binding specifically with mRNA encoding the receptor

according to the invention in the cell so as to prevent its translation. The pharmaceutical composition comprises also a pharmaceutically acceptable carrier capable of passing through said cell membrane.

5 Preferably, in said pharmaceutical composition, the oligonucleotide is coupled to a substance, such as a ribozyme, which inactivates mRNA.

10 Preferably, the pharmaceutically acceptable carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the structure. The structure of the pharmaceutically acceptable carrier in said pharmaceutical composition is capable of binding to a receptor which is specific for a selected cell type.

15 Preferably, said pharmaceutical composition comprises an amount of the antibody according to the invention effective to block the binding of a ligand to the receptor according to the invention and a pharmaceutically acceptable carrier.

20 The present invention concerns also a transgenic non human mammal overexpressing (or expressing ectopically) the nucleic acid molecule encoding the receptor according to the invention.

25 The present invention also concerns a transgenic non human mammal comprising a homologous recombination knockout of the native receptor according to the invention.

30 According to a preferred embodiment of the invention, the transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic acid according to the invention is so placed as to be transcribed into antisense mRNA which is complementary to the mRNA encoding the receptor according to the invention and

which hybridizes to mRNA encoding said receptor, thereby reducing its translation. Preferably, the transgenic non human mammal according to the invention comprises a nucleic acid molecule encoding the receptor according to the invention and comprises additionally an inducible promoter or a tissue specific regulatory element.

Preferably, the transgenic non human mammal is a mouse.

The invention relates to a method for determining whether a ligand can be specifically bound to the receptor according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting binding of ligand to such receptor and detecting the presence of any such ligand bound specifically to said receptor, thereby determining whether the ligand binds specifically to said receptor.

The invention relates to a method for determining whether a ligand can specifically bind to a receptor according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such receptor and detecting the presence of any ligand bound to said receptor, thereby determining whether the compound is capable of specifically binding to said receptor. Preferably, said method is used when the ligand is not previously known.

The invention relates to a method for determining whether a ligand is an agonist of the receptor according to the invention, which comprises contacting a cell transfected

with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting the activation of a functional receptor response from the cell and detecting by means of a bio-assay, such as a modification in a second messenger concentration or a modification in the cellular metabolism (preferably determined by the acidification rate of the culture medium), an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

The invention relates to a method for determining whether a ligand is an agonist of the receptor according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second messenger an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

The present invention relates to a method for determining whether a ligand is an antagonist of the receptor according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in second messenger concentration or a modification in the cellular metabolism, (preferably determined by the acidification rate of the culture medium) a decrease in the

receptor activity, thereby determining whether the ligand is a receptor antagonist.

The present invention relates to a method for determining whether a ligand is an antagonist of the receptor according to the invention, which comprises preparing a cell
5 extract from cells transfected with an expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand in the presence of a known receptor
10 agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second messenger, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

Preferably, the second messenger assay comprises measurement of intracellular cAMP, intracellular inositol
15 phosphate (IP3), intracellular diacylglycerol (DAG) concentration or intracellular calcium mobilization.

Preferably, the cell used in said method is a mammalian cell non neuronal in origin, such as a COS-7 cell,
20 a CHO cell, a LM(tk-) cell an NIH-3T3 cell or 1321N1.

In said method, the ligand is not previously known.

The invention is also related to the ligand isolated and detected by any of the preceding methods.

25 The present invention concerns also the pharmaceutical composition which comprises an effective amount of an agonist or an antagonist of the receptor according to the invention, effective to reduce the activity of said receptor and a pharmaceutically acceptable carrier.

30 For instance, said agonist or antagonist may be used in a pharmaceutical composition in the treatment of cystic fibrosis, and the method according to the invention

may be advantageously used in the detection of improved drugs which are used in the treatment of cystic fibrosis.

Therefore, the previously described methods may be used for the screening of drugs to identify drugs which specifically bind to the receptor according to the invention.

The invention is also related to the drugs isolated and detected by any of these methods.

The present invention concerns also a pharmaceutical composition comprising said drugs and a pharmaceutically acceptable carrier.

The invention is also related to a method of detecting expression of a receptor according to the invention by detecting the presence of mRNA coding for a receptor, which comprises obtaining total RNA or total mRNA from the cell and contacting the RNA or mRNA so obtained with the nucleic acid probe according to the invention under hybridizing conditions and detecting the presence of mRNA hybridized to the probe, thereby detecting the expression of the receptor by the cell.

Said hybridization conditions are stringent conditions.

The present invention concerns also the use of the pharmaceutical composition according to the invention for the treatment and/or prevention of cystic fibrosis.

The present invention concerns also a method for diagnosing a predisposition to a disorder associated with the activity of the receptor according to the invention. Said method comprises:

- a) obtaining nucleic acid molecules of subjects suffering from said disorder;
- b) performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;

- 5 c) electrophoretically separating the resulting nucleic acid fragments on a sized gel;
- d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to said nucleic acid molecule and labelled with a detectable marker;
- 10 e) detecting labelled bands which have hybridized to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern specific to subjects suffering from said disorder;
- f) preparing nucleic acid molecules obtained for diagnosis by step a-e; and
- 15 g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

20 A last aspect of the present invention concerns a method of preparing the receptor according to the invention, which comprises:

- 25 a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said receptor so as to permit expression thereof, wherein the cell is selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells;
- 30 b) inserting the vector of step a in a suitable host cell;
- c) incubating the cell of step b under conditions allowing the expression of the receptor according to the invention;

- d) recovering the receptor so obtained; and
- e) purifying the receptor so recovered, thereby preparing an isolated receptor according to the invention.

Short description of the drawings.

5 **Figure 1** represents nucleotide and deduced amino acid sequence of a human P2Y₄ receptor according to the invention. The putative membrane-spanning domains are underlined and numbered I to VII. The consensus sequence conserved between all the P2Y receptors and the three amino acids (AHN) corresponding to the RGD sequence in the first extracellular loop of the P2Y₂ receptor are represented in bold. The putative phosphorylation sites by PKC or by calmodulin-dependent protein kinases and PKC are indicated respectively by black squares (■) and by open circles (O).

10 **Figure 2** is a dendrogram representing structural relatedness among the cloned P2Y receptor and the closest neighbour in the G protein-coupled receptor family. The plot was constructed using the multiple sequence alignment program Pileup of the GCG package (26). For each sequence, the analysis takes into account a segment covering the first five putative membrane-spanning domains.

15 **Figure 3** represents a northern blot analysis of P2Y₄ receptor expression. The Northern blot was performed with 15 µg of total RNA from human placenta and 4 µg of poly(A)+ RNA from K562 cells and from two different human placentas. The probe was a human P2Y₄ gene fragment amplified by PCR (TM2 to TM7).

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Figure 4 represents the time course of InsP_3 accumulation in 1321N1 cells expressing the human P2Y_4 receptor. ^3H inositol labelled cells were incubated for the indicated time with UTP ($100\ \mu\text{M}$), UDP ($100\ \mu\text{M}$) and ATP ($100\ \mu\text{M}$) in the absence of $10\ \text{mM}$ LiCl (panel A) or in its presence (panel B). The data represent the mean of triplicate experimental points and are representative of two independent experiments.

Figure 5 Represents the effect of ATP on the accumulation of InsP_3 induced by UTP in 1321N1 transfected cells. Concentration-action curves of ATP in the presence of UTP 10 or $100\ \mu\text{M}$ at $30\ \text{s}$ (panel A) and $20\ \text{min}$ (panel B). Concentration-action curve of ATP with or without UTP ($10\ \mu\text{M}$) at $20\ \text{min}$ (panel C). The data represent the mean \pm S.D. of triplicate experimental points and are representative of two (panel A), five (panel B) or three (panel C) independent experiments.

Figure 6 represents the concentration-action curves of UTP and UDP on the InsP_3 accumulation in three different clones of 1321N1 transfected cells. The cells were incubated in the presence of various UTP (\bullet) and UDP (\blacksquare) concentrations (0 , 0.1 , 1 , 3 , 10 and $100\ \mu\text{M}$) for $30\ \text{s}$ or $20\ \text{min}$. The data represent the mean \pm S.D. of triplicate experimental points obtained in one representative experiment. The EC_{50} values were determined by curve fitting (Sigma Plot: version 2.0).

Figure 7 Represents the effect of various nucleotides on the InsP_3 production in 1321N1 transfected cells.

The cells were incubated with UTP, UDP, 5BrUTP, dUTP, ITP, AP₃A, AP₄A, AP₅A and AP₆A at the same concentration of 100 μ M or without agonist (Cont) for 30 s or 20 min. The data represent the mean \pm S.D. of triplicate experimental points and are representative of three independent experiments. The EC₅₀ values were determined by curve fitting (Sigma Plot: version 2.0).

Figure 8 Represents concentration-action curves of various nucleotides on the InsP₃ accumulation in 1321N1 cells expressing a human P2Y₄ receptor. 1321N1 cells were incubated in the presence of various concentrations of UTP, UDP, dUTP, 5BrUTP, ITP and ATP for a period of time of 20 min. The data are the mean \pm range of duplicate experimental points obtained in an experiment representative of two.

Figure 9 Represents the action of various P₂ antagonists on the InsP₃ production induced by UTP in 1321N1 transfected cells. Cells were incubated in the presence of suramin, reactive blue 2 and PPADS at a concentration of 100 μ M and different UTP concentrations (0, 2 and 10 μ M) for 20 min. The data represent the mean \pm S.D. of triplicate experimental points and are representative of two independent experiments.

Figure 10 Represents the effect of PPADS on the UTP stimulation of InsP₃ in 1321N1 transfected cells. The cells were exposed to various concentrations of UTP in the presence or in the absence of PPADS (100 μ M) for 20 min. The data are the mean \pm S.D. of triplicate experimental points obtained in an

experiment representative of two.

Figure 11 Represents the effect of pertussis toxin on the UTP-induced accumulation of InsP₃ in 1321N1 cells expressing a human P2Y₄ receptor. The cells were preincubated for 18 hours in the presence or in the absence of 20 ng/ml pertussis toxin. The cells were then incubated with or without UTP 100 μ M and with or without pertussis toxin (20 ng/ml) for various times: 30 s, 5 min or 20 min. The data represent the mean \pm S.D. of triplicate experimental points and are representative of two independent experiments.

Detailed description of the invention.

EXPERIMENTAL PROCEDURES

1. Materials

Trypsin was from Flow Laboratories (Bioggio, Switzerland) and the culture media, reagents, G418, fetal calf serum (FCS), restriction enzymes and Taq polymerase were purchased from GIBCO BRL (Grand Island, NY). The radioactive products myo-D-[2-³H]inositol (17.7 Ci/mmol) and [³²P]ATP (800 Ci/mmol) were from Amersham (Gent, Belgium). Dowex AG1X8 (formate form) was from Bio-Rad Laboratories (Richmond, Calif.). UTP, UDP, ATP, ADP, carbachol, LiCl and apyrase grade VII were obtained from Sigma Chemical Co. (St. Louis, MO). 2MeSATP was from Research Biochemicals Inc. (Natick, MA). pcDNA3 is an expression vector developed by Invitrogen (San Diego, CA).

2. Cloning and sequencing

Degenerate oligonucleotide primers were synthesized on the basis of the best conserved segments between the murine P2Y₂ and the chick P2Y₁ receptor sequences. These primers were used to amplify novel receptor gene fragments

by low-stringency PCR starting from human genomic DNA. The amplification conditions were as follows: 93 °C 1 min, 50 °C 2 min, 72 °C 3 min; 35 cycles. The PCR products with sizes compatible with P2 receptor gene fragments were subcloned in M13mp18 and M13mp19 and sequenced by the Sanger dideoxy nucleotide chain termination method. One of the resulting clones sharing similarities with P2 receptors, was labelled by random priming and used to screen a human genomic DNA library constructed in the λ Charon 4a vector. The hybridization was in 6 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M Sodium citrate) and 40% formamide at 42 °C for 14 h and the final wash conditions were 0.1 x SSC, 0.1% SDS at 65 °C. A preparation of λ phages (15) was made for several clones which hybridized strongly with the probe. A restriction map and a Southern blotting analysis allowed to isolate a 1.4 kb NheI-EcoRV fragment that was subcloned into the pBluescript SK⁺ vector (Stratagene). The complete sequence of a new receptor coding sequence was obtained on both strands after subcloning of overlapping fragments in M13mp18 and M13mp19.

3. Cell culture and transfection

The P2Y₄ receptor coding sequence was subcloned between the HindIII and the EcoRV sites of the pcDNA3 expression vector for transfection into 1321N1 human astrocytoma cells, a cell line which does not respond to nucleotides and which has already been used for the expression of purinergic receptors (6,12). Cells were transfected with the recombinant pcDNA3 plasmid (pcDNA3-P2Y₄) using the calcium phosphate precipitation method as described (16). 1321N1 cells were incubated for 6 hours at 37 °C in the presence of pcDNA3 vector alone or vector containing the P2Y₄ receptor coding sequence, then washed and incubated in

culture medium (10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B in Dulbecco's modified Eagle's medium (DMEM)). The selection with G418 (400 µg/ml) was started two days after transfection. From the pool
5 of transfected 1321N1 cells, individual clones were isolated by limiting dilution with the aim of selecting clones with high IP stimulation factors in response to nucleotides. The different clones were maintained in a medium containing 400 µg/ml G418.

10 4. Inositol phosphates (IP) measurement

1321N1 cells were labelled for 24 hours with 10 µCi/ml [³H] inositol in inositol-free DMEM (Dulbecco's modified Eagle's medium) medium containing 5% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml
15 amphotericin B and 400 µg/ml G418. Cells were washed twice with KRH (Krebs-Ringer Hepes) buffer of the following composition (124 mM NaCl, 5 mM KCl, 1.25 mM MgSO₄, 1.45 mM CaCl₂, 25 mM Hepes (pH 7.4) and 8 mM glucose) and incubated in this medium for 30 min. The agonists were added in the presence of LiCl (10 mM) and the incubation was stopped after
20 30 s, 5 min or 20 min by the addition of an ice-cold 3% perchloric acid solution. For the time course study, LiCl (10 mM) was added 5 min before the agonists and the incubation was stopped at different times. When tested, pertussis toxin
25 (20 ng/ml) was added for 18 h during the labelling period time and during the stimulation by the agonist. Inositol phosphates were extracted and InsP₃ was isolated by chromatography on Dowex column as described previously (17).

5. Radioligand binding assay.

30 Binding assays of [^α³²P] UTP to cell membranes were carried out in Tris-HCl (50 mM, pH 7.5), EDTA 1 mM in a final volume of 0.5 ml, containing 25-50 µg of protein and 0.5 nM

of radioligand (27). The assays were conducted at 30°C for 5 min. Incubations were stopped by the addition of 4 ml of ice-cold Tris-HCl (50 mM, pH 7.5) and rapid filtration through Whatman GF/B filters under reduced pressure. The filters were then washed three times with 2 ml of the same ice-cold Tris-HCl buffer. Radioactivity was quantified by liquid scintillation counting, after an overnight incubation of the filters in liquid scintillation mixture.

6. Northern blot and Southern blot analysis

Total and poly(A)⁺ RNA were prepared from different tissues and human cell lines using the guanidinium thiocyanate-caesium chloride procedure (15), denatured by glyoxal and fractionated by electrophoresis on a 1% agarose gel in 10 mM phosphate buffer pH 7.0. DNA samples, prepared from the λ Charon 4a clones, were digested with restriction enzymes. Northern and Southern blots were prepared (15) and baked for 90 min at 80 °C. Membranes were prehybridized for at least 4 hours and hybridized overnight with the same probe as for the screening, at 42 °C in a solution containing 50% formamide for Northern blots and 40% formamide for Southern blots. Filters were washed twice for 15 min in 2 x SSC at room temperature and then twice for 30 min in 0.2 x SSC at 60 °C before being exposed at -70 °C in the presence of intensifying screens for 5 days (Northern blots) or 1 hour (Southern blots).

RESULTS

1. Cloning and sequencing

In order to isolate new subtypes of P2 receptors, sets of degenerate oligonucleotide primers were synthesized on the basis of the best conserved segments in the published sequences of the chick brain P2Y1 (5) and murine

neuroblastoma P2Y2 (9) receptors. These primers were used in low-stringency PCR on human genomic DNA as described (18). Some combinations generated discrete bands with a size compatible with that expected for P2 receptors. For example, the primer [5'-CAGATCTAGATA(CT)ATGTT(CT)(AC)A(CT)(CT)T(ACGT)GC-3'] corresponding to the second transmembrane region and the primer [5'-TCTTAAGCTTGG(AG)TC(ACGT)A(CG)(AG)CA(AG)CT(AG)TT-3'] corresponding to the seventh transmembrane region amplified a 712 bp fragment. The partial sequences obtained after sequencing were translated into peptidic sequences and compared to a local databank which contains G protein-coupled receptor sequences. Most of the clones resulting from these PCR products encoded a part of a new receptor which displayed 58% identity with the murine P2Y2 receptor and 42% identity with the chick P2Y1 receptor partial sequences. In addition, some clones encoded a peptidic sequence presenting 87% identity with the chick P2Y1 receptor and are therefore believed to represent fragments of the human P2Y1 gene.

The partial sequence of the new receptor was used as a probe to screen a human genomic DNA library. Several clones that strongly hybridized with the probe at high stringency conditions were obtained and purified. The inserts of the clones varied from 12 to 17 kb and restriction analysis revealed that all clones belonged to a single locus. The full sequence of a 1.4 kb NheI-EcoRV fragment was obtained and an intronless open reading frame of 1095 bp was identified. The sequence is depicted in figure 1 where the putative membrane-spanning domains are underlined and numbered I to VII. The predicted molecular weight of the encoded protein is 36.5 kDa. This molecular weight is unlikely to be modified in vivo, since no N-glycosylation consensus sequences are found in the putative exofacial

regions. In contrast with the human P2Y2 receptor, there is no RGD motif, an integrin binding consensus sequence, in the putative first extracellular loop. The three amino acid (AHN) corresponding to the RGD sequence in the first extracellular loop of the P2Y2 receptor are represented in bold in figure 1. Some potential sites of phosphorylation by protein kinase C (PKC) or by calmodulin-dependent protein kinases were identified in the third intracellular loop and in the carboxyterminal part of the receptor. The putative phosphorylation sites by PKC or by calmodulin-dependent protein kinases and PKC are indicated respectively by black squares and by open circles in figure 1. The four positively charged amino acid which have been reported to play a role in the P2Y2 receptor activation by ATP and UTP (1) are conserved in the P2Y4 sequence: His²⁶², Arg²⁶⁵, Lys²⁸⁹ and Arg²⁹² (Figure 1). The P2Y4 amino acid sequence was compared to the chick P2Y1 and the murine P2Y2 amino acid sequences and to their closest neighbours in the G protein-coupled receptor family (Figure 2). The plot was constructed using the multiple sequence alignment program Pileup of the GCG package (26). For each sequence, the analysis takes into account a segment covering the first five putative membrane-spanning domains. It is clear that, from a structural point of view, the newly cloned receptor is more closely related to the human P2Y2 receptor (51% of identity between the complete sequences) than to the chick P2Y1 receptor (35%).

2. Tissue distribution of the P2Y4 receptor

The tissue distribution of P2Y4 transcripts was investigated by Northern blotting. A number of rat tissues (heart, brain, liver, testis and kidney) were tested using a human probe at low stringency, but no hybridization signal could be obtained. No P2Y4 transcript could be detected in

the following human cell lines: K562 leukemia cells (Figure 3), HL-60 leukemia cells and SH-SY5Y human neuroblastoma cells. The Northern blot was performed with 15 μ g of total RNA from human placenta and 4 μ g of poly(A)⁺ RNA from K562 cells and from two different human placentas. The probe was the human P2Y₄ gene fragment amplified by PCR (TM2 to TM7). On the contrary, a strong signal, corresponding to a 1.8 kb mRNA, was found in human placenta (Figure 3).

3. Functional expression of the new P2 receptor in 1321N1 cells

After transfection of the pcDNA3-P2Y₄ construction in 1321N1 cells, the pool of G418-resistant clones was tested for their functional response (IP₃ accumulation) to ATP and UTP. Both nucleotides were found to be agonists of the P2Y₄ receptor, but the response to UTP was more robust. About 20 transfected clones were then isolated and tested for their response to UTP. The clone presenting the highest IP accumulation factor in response to UTP was selected and used in all subsequent experiments. Functional characterization of the P2Y₄ receptor was performed by determining the accumulation of InsP₃ after 20 min incubation with the agonists in the presence of 10 mM LiCl. We observed that the response to UTP was biphasic, with a peak reached at 30 s, followed by a more sustained stimulation of lower magnitude (Fig. 4A). With ATP, only that second phase was detectable: its effect became apparent after 1 min of stimulation only and was stable for at least 20 min (Fig. 4A and B). As for UTP, the stimulation by UDP was biphasic, but it was slightly delayed (Fig 4A and B). Inclusion of LiCl had little effect on the initial peak induced by UTP or UDP, but it strongly enhanced the following plateau phase (Fig. 4B).

The maximal effect of ATP observed after a 20 min

incubation represented about $27 \pm 9\%$ of that of UTP (mean \pm S.D. of ten experiments). In order to demonstrate that ATP is able to antagonize the UTP response, incubations of 1321N1 cells were conducted with ATP alone or in combination with UTP. Figure 5 shows that at high concentration (500 μ M or more), ATP was able to inhibit the effect of UTP, both at 30 s and 20 min. At 30 s, the response to UTP 10 μ M was fully antagonized by ATP 2 mM, corresponding to the fact that ATP has no effect on the human P2Y₄ receptor at this early time (panel A). At 20 min, an inhibition of $62 \pm 11\%$ of the UTP effect (10 μ M), corresponding to the difference between the UTP and the ATP effects, was observed in the presence of 2 mM ATP (mean \pm S.D. of five independent experiments) (panels B and C). The ATP concentration-inhibition curves were shifted to the right when the UTP concentration was increased, indicating the competitive nature of this inhibitory effect (panels A and B). On the other hand, at lower concentrations (30-300 μ M), ATP enhanced the response to UTP by 29% (range 12-47%, mean of four experiments) (panel B). ADP, which had almost no effect per se and did not inhibit the action of UTP, reproduced that enhancement: in the presence of ADP (100 μ M), the stimulation by UTP (10 μ M) represented $158 \pm 15\%$ (mean of three independent experiments) of that by UTP alone (data not shown). However, this potentiating effect of ATP and ADP was not specific: indeed the action of carbachol mediated by muscarinic receptors endogenously expressed in the 1321N1 cells (6) was also increased in the presence of these nucleotides. This observation was reproduced with cells transfected with the recombinant P2Y₄-pcDNA3 plasmid or with the vector alone and was also obtained with AMP and adenosine (data not shown).

We compared the concentration-action curves of UTP

and UDP on the InsP_3 production for several clones of transfected cells. The study was made at two times (Fig. 6) : 30 s and 20 min. In the set of experiments performed on clone 11 (clone of 1321N1 transfected cells chosen for the pharmacological characterization), UTP appeared to be 10-fold more potent than UDP after a 20 min incubation and this difference was reproduced with two other clones (Fig. 6). The EC_{50} values were $0.3 \pm 0.1 \mu\text{M}$ and $3.3 \pm 0.6 \mu\text{M}$ in clone 2, $2.4 \pm 0.1 \mu\text{M}$ and $19.8 \pm 4.8 \mu\text{M}$ in clone 11 and $0.3 \pm 0.1 \mu\text{M}$ and $3.2 \pm 0.8 \mu\text{M}$ in clone 21, respectively, for UTP and UDP (mean \pm S.D. of two independent experiments). At 30 s of incubation, it was not possible to determine EC_{50} values because the curves were clearly shifted to the right, but we can observe that the difference between the two agonists potency was even more striking (Fig. 6). Several clones, including clones 2, 11 and 21 were tested in binding studies with $[\alpha^{32}\text{P}]$ UTP but no increase in specific binding was observed as compared to the cells transfected with the vector alone (data not shown).

In view of the time differences observed in Figure 6, the testing of a range of nucleotides was performed at two times: 30 s and 20 min. As Figure 7 shows, several agonists were barely or not active at 30 s (UDP, 5BrUTP, dUTP, ITP) whereas they produced a significant effect at 20 min. Full concentration-action curves were obtained at 20 min. The rank order of potency was: $\text{UTP} > \text{UDP} = \text{dUTP} > 5\text{BrUTP} > \text{ITP} > \text{ATP}$ (Fig. 8). The EC_{50} values obtained were the following: EC_{50} UTP = $2.5 \pm 0.6 \mu\text{M}$, EC_{50} UDP = $19.5 \pm 3.9 \mu\text{M}$ (mean \pm S.D. of eight independent experiments), EC_{50} dUTP = $20.0 \pm 2.3 \mu\text{M}$, EC_{50} 5BrUTP = $27.1 \pm 1.9 \mu\text{M}$ and EC_{50} ITP = $32.8 \pm 5.4 \mu\text{M}$ (mean \pm S.D. of two independent experiments). The approximative EC_{50} value obtained for ATP was: $43 \pm 12 \mu\text{M}$ (mean \pm S.D. of five

independent experiments). The diadenosine polyphosphates also increased the InsP_3 production in transfected cells with EC_{50} between 3 and 7 μM (data not shown), but their maximal effect was only 20-25 % of that of UTP, a value close to that of ATP (range of four independent experiments) (Fig. 7). UMP, uridine, AMP, adenosine and $\text{ATP}\gamma\text{S}$ were without any effect (data not shown).

No specific antagonist is available for any P_2Y subtype. Nonetheless, several non-selective antagonists such as suramin, RB2 or PPADS have been tested on P_2 receptors and their relative actions on these subtypes may constitute a mean to discriminate them (27). So we tested the ability of these three antagonists to inhibit the UTP response in the model of the human P_2Y_4 receptor. As we can see on figure 9, PPADS appeared to be the most active antagonist ($73 \pm 14\%$ inhibition; IC_{50} around 15 μM (data not shown)), suramin was inactive, and RB-2 produced an inhibition of $33 \pm 5\%$ of the UTP response (mean \pm S.D. of two independent experiments). Figure 10 shows the mixed nature of the antagonism by PPADS of the UTP response: it affects both the EC_{50} value and the maximal effect of UTP. The EC_{50} value for UTP in the absence of PPADS was $3.3 \pm 0.6 \mu\text{M}$ and $12.2 \pm 4.5 \mu\text{M}$ in the presence of 100 μM PPADS (mean \pm S.D. of two independent experiments).

The effect of pertussis toxin (20 ng/ml, 18 hours pretreatment) was studied at different times after UTP (100 μM) addition (Fig. 11). The UTP response was clearly inhibited at 30 s ($62 \pm 5\%$ of inhibition: mean \pm S.D. of two independent experiments), whereas no significant effect was observed at 5 and 20 min.

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CLAIMS

- 5 1. Receptor which has an amino acid sequence having more than 50% homology with the amino acid sequence shown in Figure 1.
2. Receptor according to claim 1, which has the amino acid sequence shown in Figure 1.
- 10 3. Receptor according to claim 1 or 2 having a preference for pyrimidine nucleotides over purine nucleotides.
4. Receptor according to claim 3, having at least a twofold preference, preferably tenfold to one
15 hundredfold preference for pyrimidine nucleotides over purine nucleotides.
5. Receptor according to any of the claims 3 or 4, wherein the pyrimidine nucleotide is uridine triphosphate.
- 20 6. Receptor according to any of the claims 3 to 5, having a preference for UTP over UDP.
7. Receptor according to claim 5 being a high affinity UTP-specific receptor.
8. Receptor according to any of the preceding
25 claims, belonging to the P2 receptor family.
9. Receptor according to any of the preceding claims, being a G protein-coupled receptor.
10. Receptor according to any of the preceding claims, being a human receptor.
- 30 11. Nucleic acid molecule encoding the receptor according to any of the preceding claims.

12. Nucleic acid molecule according to claim 11, wherein the nucleic acid molecule is DNA or RNA molecule.

13. DNA molecule according to claim 12, which
5 is a cDNA molecule or a genomic DNA molecule.

14. Nucleic acid molecule according to any of the claims 11 to 13, having more than 50% homology to the DNA sequence shown in Figure 1.

15. DNA molecule according to claim 14, which
10 has the DNA sequence shown in figure 1.

16. Vector comprising the nucleic acid molecule according to any of the claims 11 to 15.

17. Vector according to claim 16, adapted for expression in a cell, which comprises the regulatory
15 elements necessary for expression of the nucleic acid molecule in said cell operatively linked to the nucleic acid molecule according to any of the claims 11 to 15 as to permit expression thereof.

18. Vector of claim 17, wherein the cell is
20 chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells.

19. Vector according to any of the claims 16 to 18, wherein the vector is a plasmid or a virus.

20. Vector according to claim 19, being a
25 virus selected from the group consisting of baculovirus, adenovirus or Semliki Forest virus.

21. Cell comprising the vector according to any of the claims 16 to 20.

22. Cell of claim 21, wherein the cell is a
30 mammalian cell, preferably non neuronal in origin.

AMENDED SHEET

23. Cell of claim 21, wherein the cell is chosen among the group consisting of COS-7 cells, LM(tk-) cells, NIH-3T3 cells or 1321N1 cells.

24. Nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridising with a unique sequence included within the nucleic acid molecule according to any of the claims 11 to 15.

25. Nucleic acid probe of claim 24, wherein the nucleic acid is DNA or RNA.

26. Antisense oligonucleotide having a sequence capable of specifically hybridising to a mRNA molecule of claim 12, so as to prevent translation of the mRNA molecule.

27. Antisense oligonucleotide having a sequence capable of specifically hybridising to the DNA molecule of claim 13.

28. Antisense oligonucleotide according to claim 26 or 27, comprising chemical analogs of nucleotides.

29. Ligand other than purine and pyridine nucleotides capable of binding to a receptor according to any of the claims 1 to 10.

30. Anti-ligand capable of competitively inhibiting the binding of the ligand according to claim 29 to the receptor according to any of the claims 1 to 10.

31. Ligand according to claim 29 which is an antibody.

32. Anti-ligand according to claim 30 which is an antibody.

33. Antibody according to claim 31 or 32, which is a monoclonal antibody.

34. Monoclonal antibody according to claim 33, directed to an epitope of the receptor according to any of the claims 1 to 10, present on the surface of a cell expressing said receptor.

5 35. Pharmaceutical composition comprising an amount of the oligonucleotide according to claim 26, effective to decrease activity of the receptor according to any of the claims 1 to 10 by passing through a cell membrane and binding specifically with mRNA encoding said
10 receptor in the cell so as to prevent its translation, and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

36. Pharmaceutical composition of claim 35, wherein the oligonucleotide is coupled to a substance which
15 inactivates mRNA.

37. Pharmaceutical composition of claim 36, wherein the substance which inactivates mRNA is a ribozyme.

38. Pharmaceutical composition according to any of the claims 35 to 37, wherein the pharmaceutically
20 acceptable carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the structure.

39. Pharmaceutical composition of claim 38, wherein the structure of the pharmaceutically acceptable
25 carrier is capable of binding to a receptor which is specific for a selected cell type.

40. Pharmaceutical composition which comprises an effective amount of the anti-ligand of claim 30, effective to block binding of a ligand to the receptor
30 according to any of the claims 1 to 10 and a pharmaceutically acceptable carrier.

41. Transgenic non human mammal expressing the nucleic acid molecule according to any of the claims 11 to 15.

42. Transgenic non human mammal comprising a homologous recombination knockout of the native receptor according to any of the claims 1 to 10.

43. Transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic acid molecule according to any of the claims 11 to 15 so placed as to be transcribed into antisense mRNA which is complementary to the mRNA of claim 12 and which hybridises to said mRNA thereby reducing its translation.

44. Transgenic non human mammal according to any of the claims 41 to 43, wherein the nucleic acid according to any of the claims 11 to 15 additionally comprises an inducible promoter.

45. Transgenic non human mammal according to any of the claims 41 to 43, wherein the nucleic acid according to claim 11 to 15 additionally comprises tissue specific regulatory elements.

46. Transgenic non human mammal according to any of the claims 41 to 45, which is a mouse.

47. Method for determining whether a ligand can specifically bind to a receptor according to any of the claims 1 to 10, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting binding of ligand to such receptor and detecting the presence of any such ligand bound specifically to said receptor, thereby determining whether the ligand binds specifically to said receptor.

48. Method for determining whether a ligand can specifically bind to the receptor according to any of the claims 1 to 10, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such receptor and detecting the presence of any ligand bound to said receptor, thereby determining whether the compound is capable of specifically binding to said receptor.

49. Method for determining whether a ligand is an agonist of the receptor according to any of the claims 1 to 10, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting the activation of a functional receptor response from the cell and detecting by means of a bio-assay, such as a modification in a second messenger concentration or a modification in the cellular metabolism, an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

50. Method for determining whether a ligand is an agonist of the receptor according to any of the claims 1 to 10, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second messenger, an increase in the

receptor activity, thereby determining whether the ligand is a receptor agonist.

51. Method for determining whether a ligand is an antagonist of the receptor according to any of the
5 claims 1 to 10, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response
10 and detecting by means of a bio-assay, such as a modification in a second messenger concentration or a modification in the cellular metabolism, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

52. Method for determining whether a ligand is an antagonist of the receptor according to any of the
claims 1 to 10, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane
20 fraction from the cell extract, contacting the membrane fraction with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in a second
25 messenger concentration, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

53. A method according to any of the claims 47 to 50, wherein the second messenger assay comprises
30 measurement of intra-cellular cAMP, intra-cellular Inositol phosphate, intra-cellular diacylglycerol concentration or intra-cellular calcium mobilisation.

54. Method according to any of the claims 47 to 53, wherein the cell is a mammalian cell, preferably non neuronal in origin, and chosen among the group consisting of COS-7 cells, CHO cells, LM(tk-) cells, NIH-3T3 cells or 1321N1 cells.

55. Method according to any of the claims 47 to 54, wherein the ligand is not previously known.

56. Ligand detected by the method according to any of the preceding claims 47 to 55.

57. Pharmaceutical composition which comprises the ligand according to claim 56 and a pharmaceutically acceptable carrier.

58. Method of detecting the expression of the receptor according to any of the claims 1 to 10, by detecting the presence of mRNA coding said receptor, which comprises obtaining total RNA or total mRNA from the cell and contacting the RNA or mRNA so obtained with the nucleic acid probe according to claim 23 under hybridising conditions, and detecting the presence of mRNA hybridised to the probe, thereby detecting the expression of the receptor by the cell.

59. Method of detecting the presence of the receptor according to any of the claims 1 to 10 on the surface of a cell, which comprises contacting the cell with the antibody of claim 31 under conditions permitting binding of the antibody to the receptor, and detecting the presence of the antibody bound to the cell, thereby detecting the presence of the receptor on the surface of the cell.

60. Method of determining the physiological effects of expressing varying levels of the receptor according to any of the claims 1 to 10, which comprises

producing a transgenic non human mammal according to any of the claims 41 to 46 whose levels of receptor expression are varied by use of an inducible promoter which regulates the receptor expression.

5 61. Method of determining the physiological effects of expressing varying levels of the receptor according to any of the claims 1 to 10, which comprises producing a panel of transgenic non human mammals according to any of the claims 41 to 46, each expressing a different
10 amount of said receptor.

 62. Method for identifying an antagonist of the receptor according to any of the claims 1 to 10 capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of the
15 receptor, which comprises administering the antagonist to a transgenic non human mammal according to any of the claims 41 to 46 and determining whether the antagonist alleviates the physical and behavioural abnormalities displayed by the transgenic non human mammal as a result of receptor
20 activity, thereby identifying the antagonist.

 63. Antagonist identified by the method of claim 62.

 64. Pharmaceutical composition comprising an antagonist according to claim 63 and a pharmaceutically
25 acceptable carrier.

 65. Method for identifying an agonist of the receptor according to any of the claims 1 to 10 capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by activation of said receptor,
30 which comprises administering the agonist to a transgenic non human mammal according to any of the claims 41 to 46 and determining whether the antagonist alleviates the

physical and behavioural abnormalities displayed by the transgenic non human mammal, the alleviation of the abnormalities indicating the identification of the agonist.

5 65. 66. Agonist identified by the method of claim

67. Pharmaceutical composition comprising an agonist according to claim 66 and a pharmaceutically acceptable carrier.

10 68. Method for diagnosing a predisposition to a disorder associated with the activity of a specific allele of the receptor according to any of the claims 1 to 10, which comprises :

- 15 a) obtaining nucleic acid molecules of subjects suffering from said disorder;
- b) performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;
- c) electrophoretically separating the resulting nucleic acid fragments on a sized gel;
- 20 d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridising to said nucleic acid molecule and labelled with a detectable marker;
- e) detecting labelled bands which have hybridised to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern specific to
- 25 subjects suffering from said disorder;
- f) preparing nucleic acid molecules obtained for diagnosis by step a-e; and
- 30 g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose

thereby predisposition to the disorder if the patterns are the same.

69. Method of preparing the purified receptor according to any of the claims 1 to 10, which comprises :

- 5 a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said receptor so as to permit expression thereof,
- 10 wherein the cell is selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells;
- b) inserting the vector of step a in a suitable host cell;
- c) incubating the cell of step b under conditions allowing
- 15 the expression of the receptor according to the invention;
- d) recovering the receptor so obtained; and
- e) purifying the receptor so recovered, thereby preparing an isolated receptor according to the invention.

ENCLOSED SHEET

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	AAGGGAGCTTGGGTAGGGGCCAGGCTAGCCTGAGTGCACCCAGATGCGCTTCTGTGAGCT	60
	CTCCCTAGTGCTTCAACCACTGCTCTCCCTGCTCTACTTTTGTGCTCCAGCTCAGGGAT	120
	GGGGGTGGGCAGGGAAATCCTGCCACCCCTCACTTCTCCCTTCCCATCTCCAGGGGGGCC	180
1	ATGGCCAGTACAGAGTCTCTCCCTGTTGAGATCCCTAGGCCCTCAGCCCAGGTCTCTGGCAGC	240
1	M A S T E S S L L R S L G L S P G P G S	
61	AGTGAGGTGGAGCTGGACTGTTGGTTTGATGAGGATTTCAAGTTCATCCTGCTGCCCTGTG	300
21	S E V E L D C W F D E D F K F I L L P V	
121	AGCTATGCAGTTGTCTTTGTGCTGGGCTTGGGCCCTTAACGCCCCCAACCCATATGGCTCTTC	360
41	S Y A V V F V L G L G L N A P T L W L F	
181	ATCTTCCGCTCCGACCCCTGGGATGCAACGGCCACCTACATGTTCCACCTGGCATTTGTCA	420
61	I F R L R P W D A T A T Y M F H L A L S	
241	GACACCTTGTATGTGCTGTGCTGCCACCCCTCATCTACTATTATGCAGCCCAACAC	480
81	D T L V L S L P T L I V Y A A H N H	
301	TGGCCCTTGGCACTGAGATCTGCAAGTTGCTCCGCTTCTCTTTCTATTGGAACCTCTAC	540
101	W P F G T E I C K F V R F L F V W N L Y	
361	TGCAGTGTCTTTTCTCCTCACCTGCATCAGCGTGCACCGCTACCTGGGCATCTGCCACCCA	600
121	C S V L F L T C I S V H R Y L G I C H P	
421	CTTCGGGCACTACGCTGGGGCCGCCCTCGCCTCGCAGGCCCTCTCTGCCCTGGCAGTTTGG	660
141	L R A L R W G R P R L A G L L C L A V W	
481	TTGGTCTGTAGCCGGCTGCCTCGTGCCTCAACCTGTTCTTTGTGCACAACCAGCAACAAAGGG	720
161	L V V A G C L V P N L F F V T T S N K G	
541	ACCACCTCTCTGTGCCATGACACCACTCGGCCCTGAAGAGTTTGACCCTATGTGCACTTC	780
181	T T V L C H D T T R P E E F D H Y V H F	
601	AGCTCGGCGGTTCATGGGGCTGCTCTTTGGCGTGCCTGCTGGTCACTCTTGTGCTAT	840
201	S S A V M G L L F G V P C L V T V L V	
661	GGACTCATGGCTCGTGCCTGTATCAGCCCTTGGCAGGCTCTGCACAGTCTGCTTCTCGC	900
221	G L M A R R L Y Q P L P G S A Q S S S R	
721	CTCCGCTCTCTCCGACCATAGCTGTGGTGCTGACTGTCTTTGCTGTCTGCTGCTGCT	960
241	L R S L R T L A V V L T V F A V C F V	
781	TTCCACATCACCCGACCATTTACTACCTGGCCAGGCTGTTGGAAGCTGACTGCCGAGTA	1020
261	F H T R T L Y V L A R L L E A D C R V	
841	CTGAACATTGTCAACGTGGTCTATAAAGTGACTCGGCCCTGGCCAGTGCCAACAGCTGC	1080
281	L N I V N V V Y K V T R P L A S A N S C	
901	CTGGATCCTGTGCTCTACTTGCTCACTGGGGACAAATATCGACGTCAGCTCCGTCAGCTC	1140
301	L D P V L Y L L T G D K Y R R Q L R Q L	
961	TGTGGTGGTGGCAAGCCCCAGCCCCGACGGCTGCCTCTTCCCTGGCACTAGTGTCCCTG	1200
321	C G G G K P Q P R T A A S S L A L V S L	
1021	CCTGAGGATAGCAGCTGCAGGTGGGCGGCCACCCCCAGGACAGTAGCTGCTCTACTCCT	1260
341	P E D S S C R W A A T P Q D S S C S T P	
1081	AGGGCAGATAGATTGTAACACGGGAAGCCGGGAAGTGAGAGAAAAGGGGATGAGTGCAGG	1320
361	R A D R L *	
	GCAGAGGTGAGGGAACCCCAATAGTGATACCTGGTAAGGTGCTCTTCTCTTTTCCAGGC	1380
	TCTGGAGAGAAGCCCTCACCCCTGAGGGTTGCCAGGGAGGCAGGGATATC	1429

FIG. 1

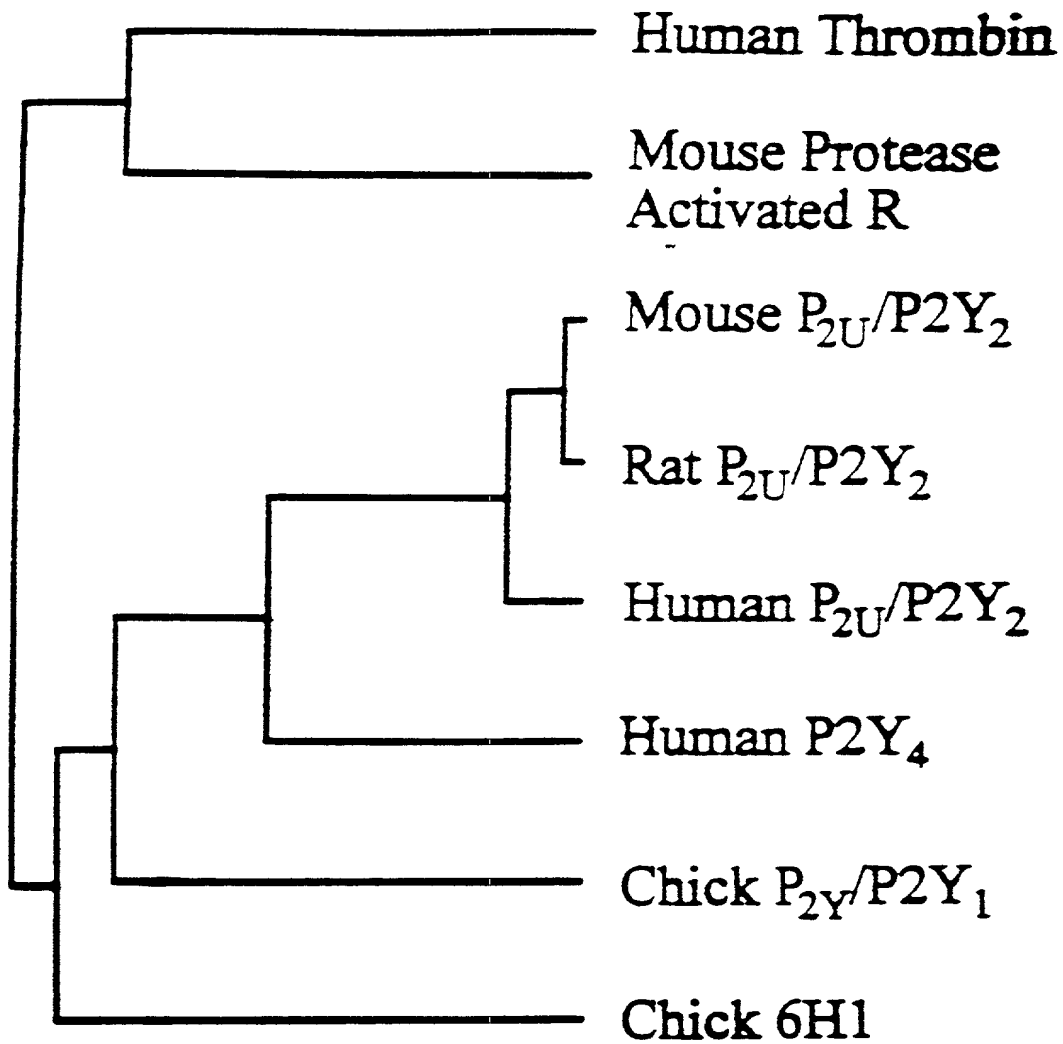
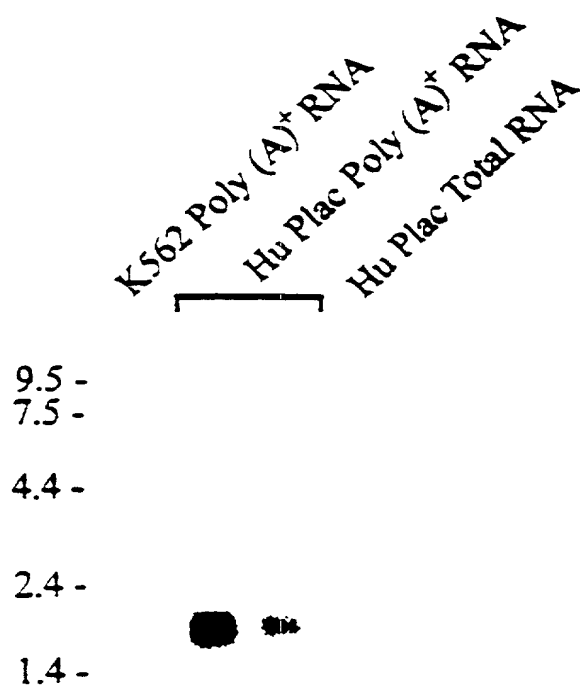


FIG. 2

Expression of P2Y₄ receptorsFIG.3

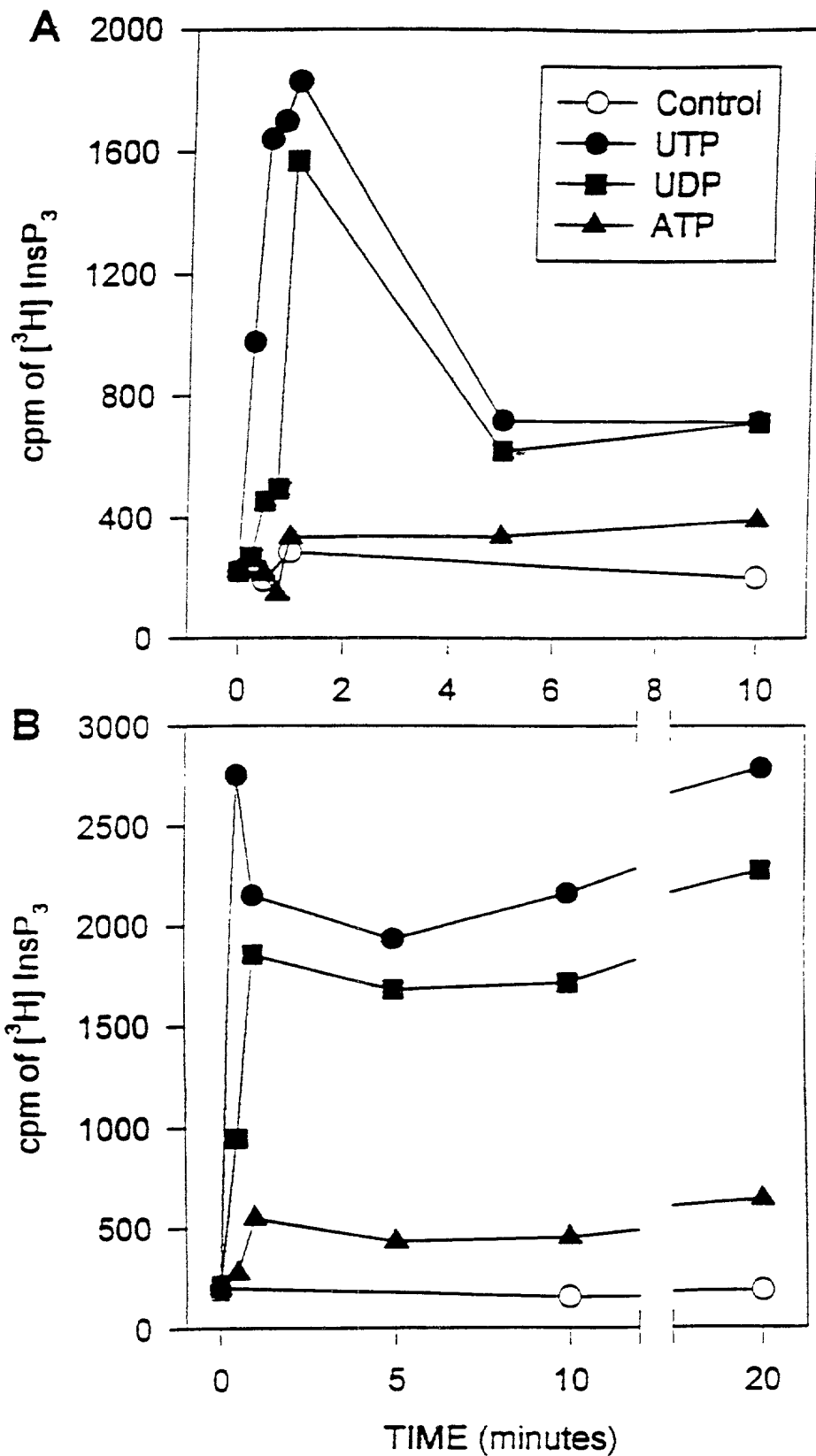


Fig. 4

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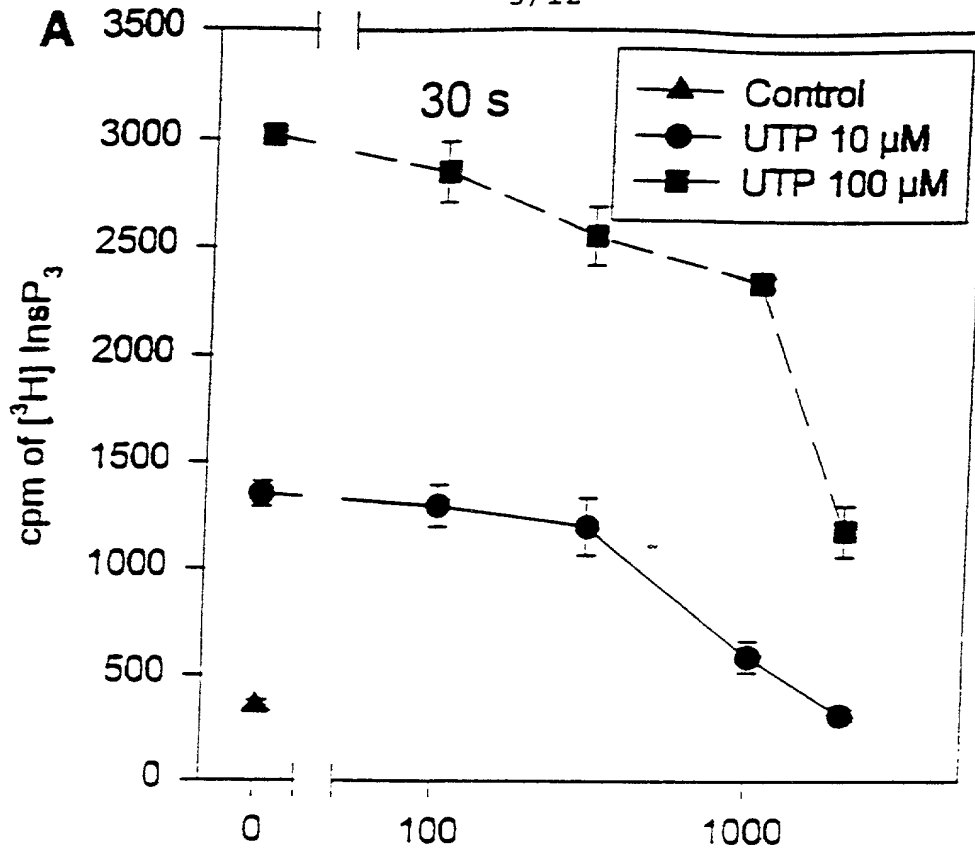


Fig. 5
Panel A

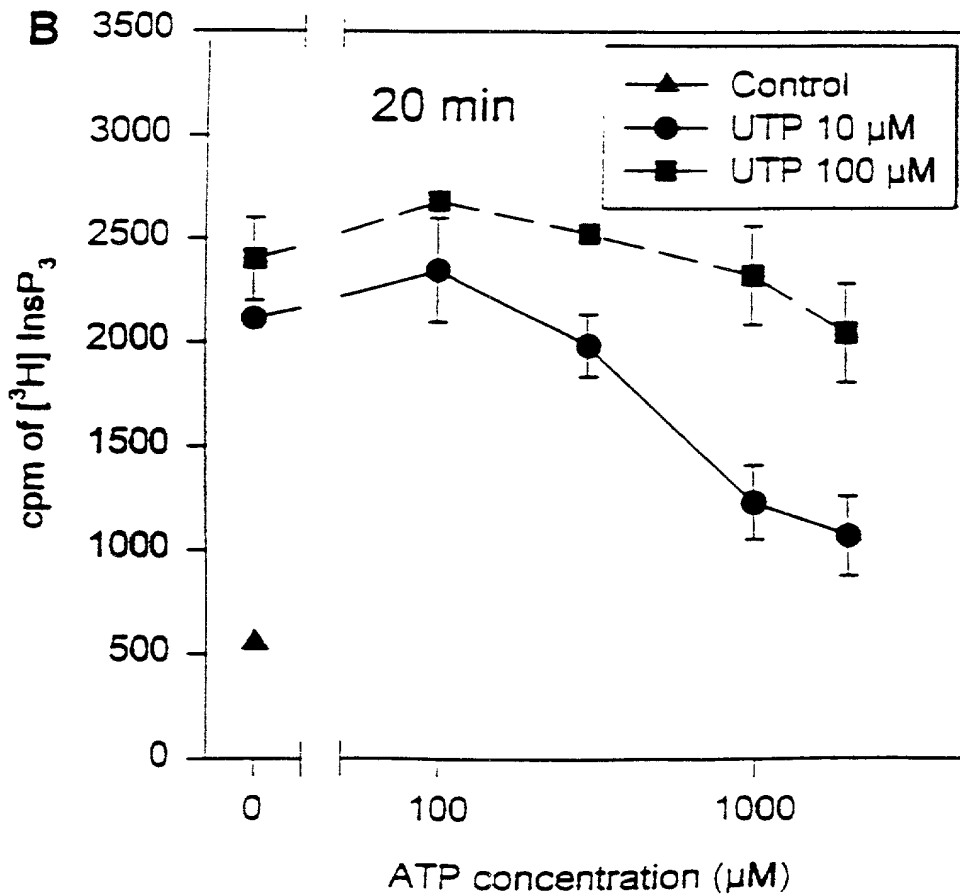
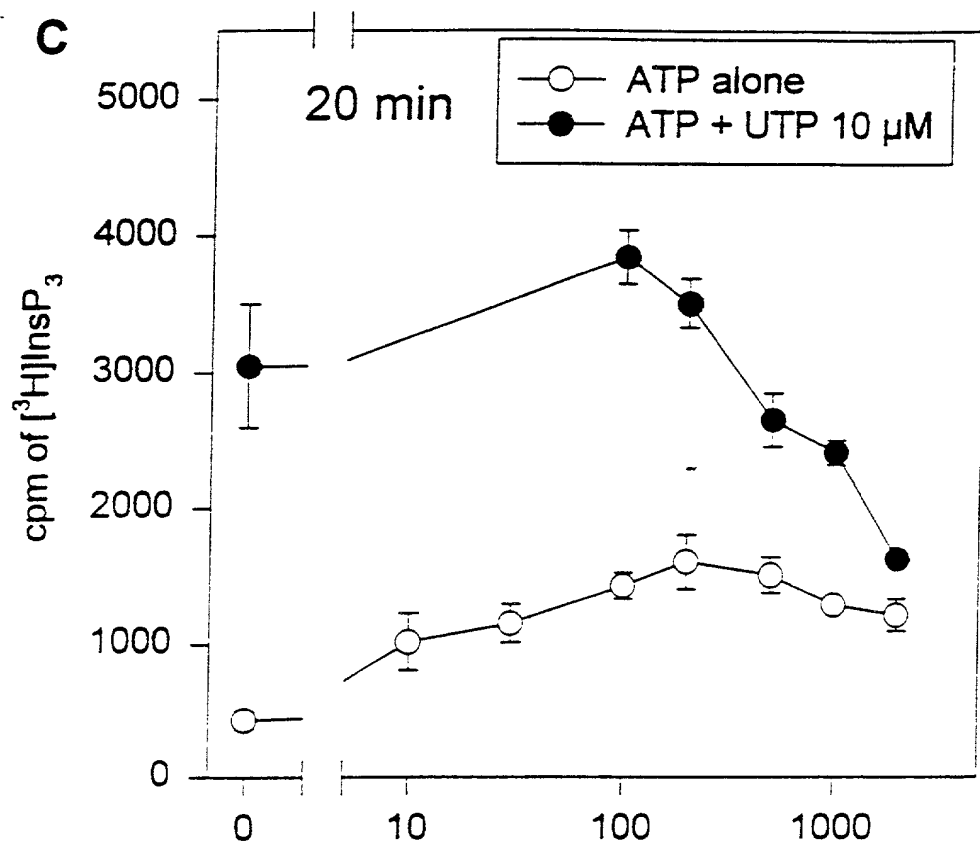


Fig. 5
Panel B

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Fig. 5
Panel C.

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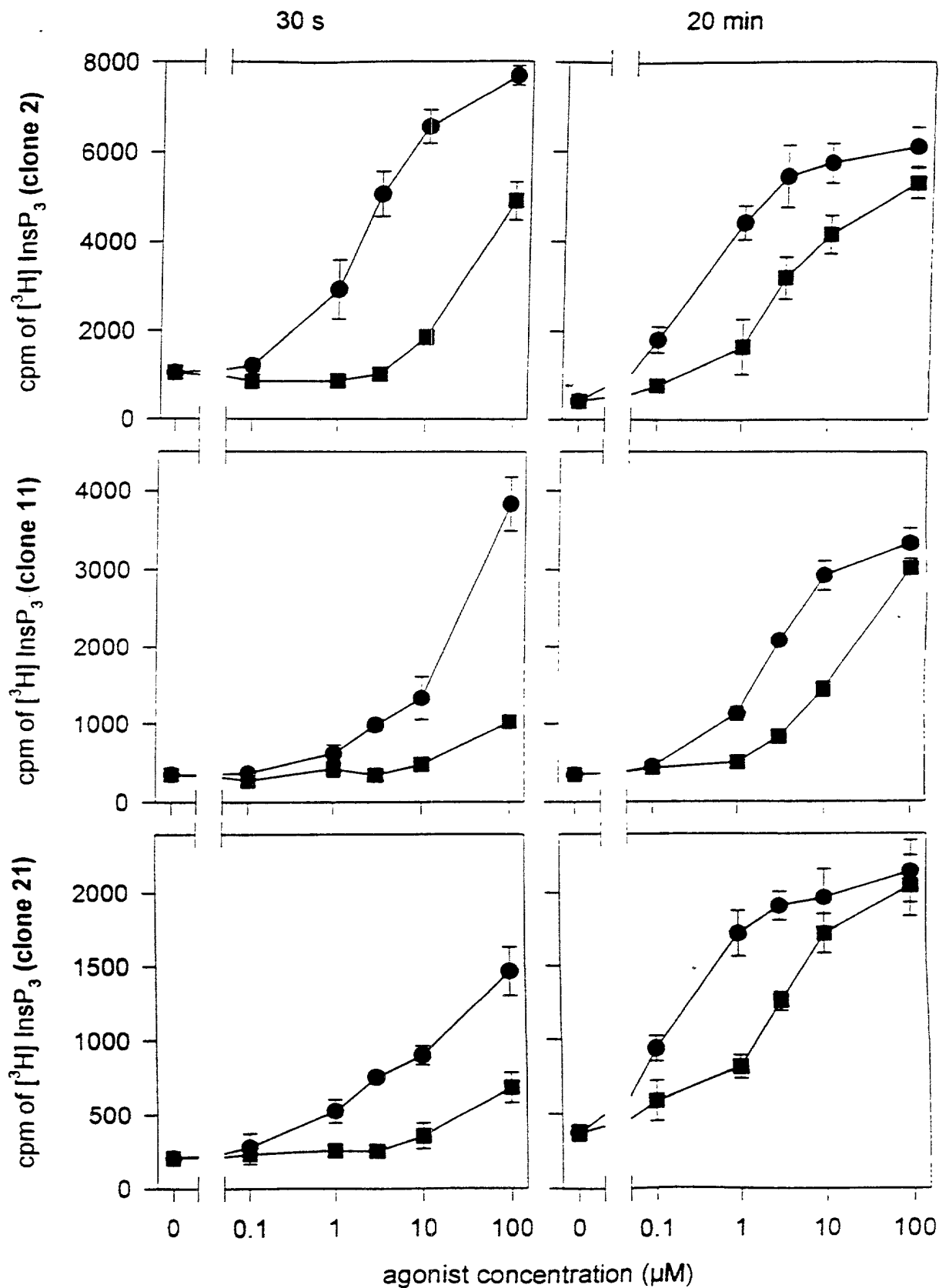


Fig.6

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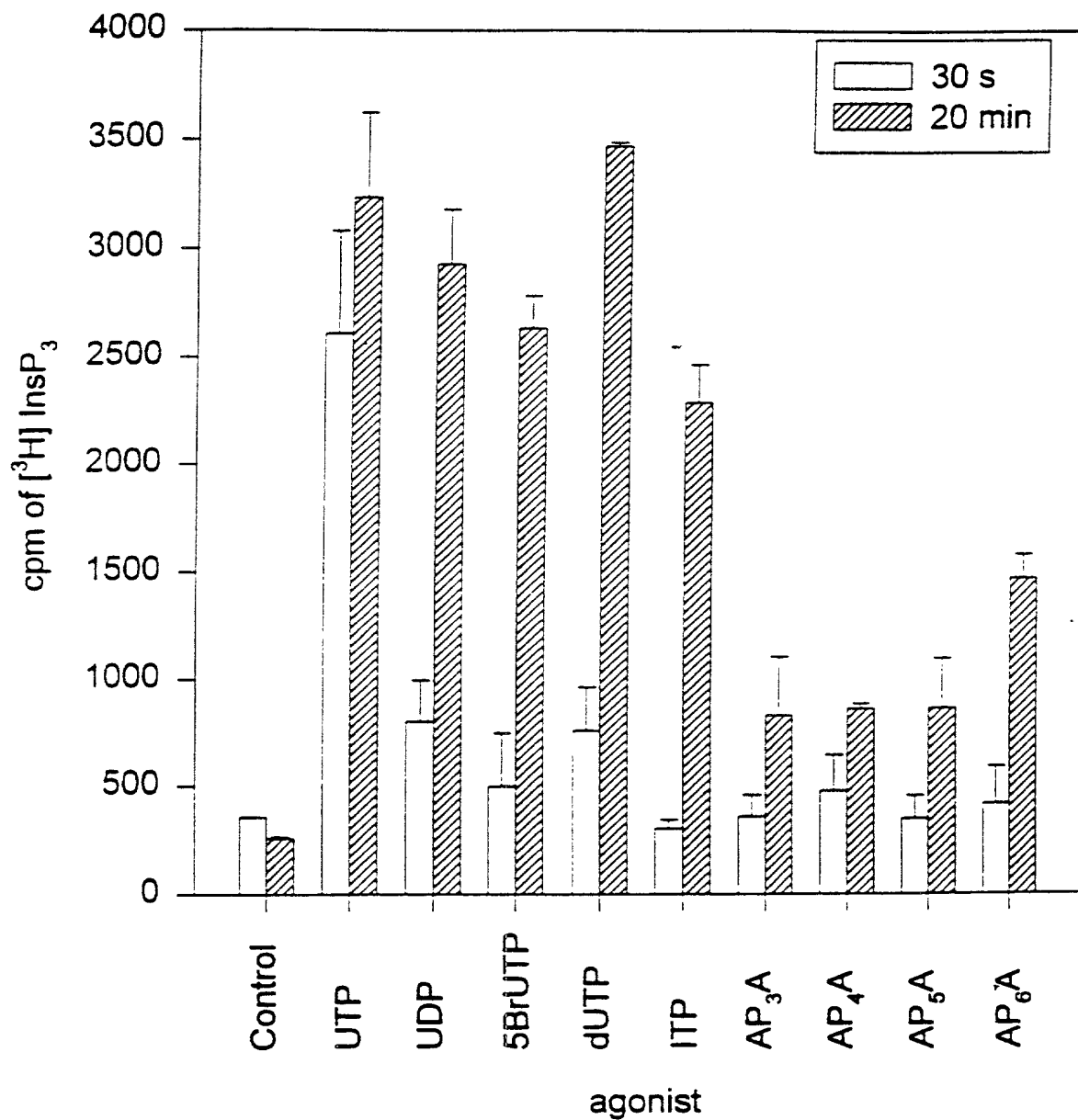


Fig. 7

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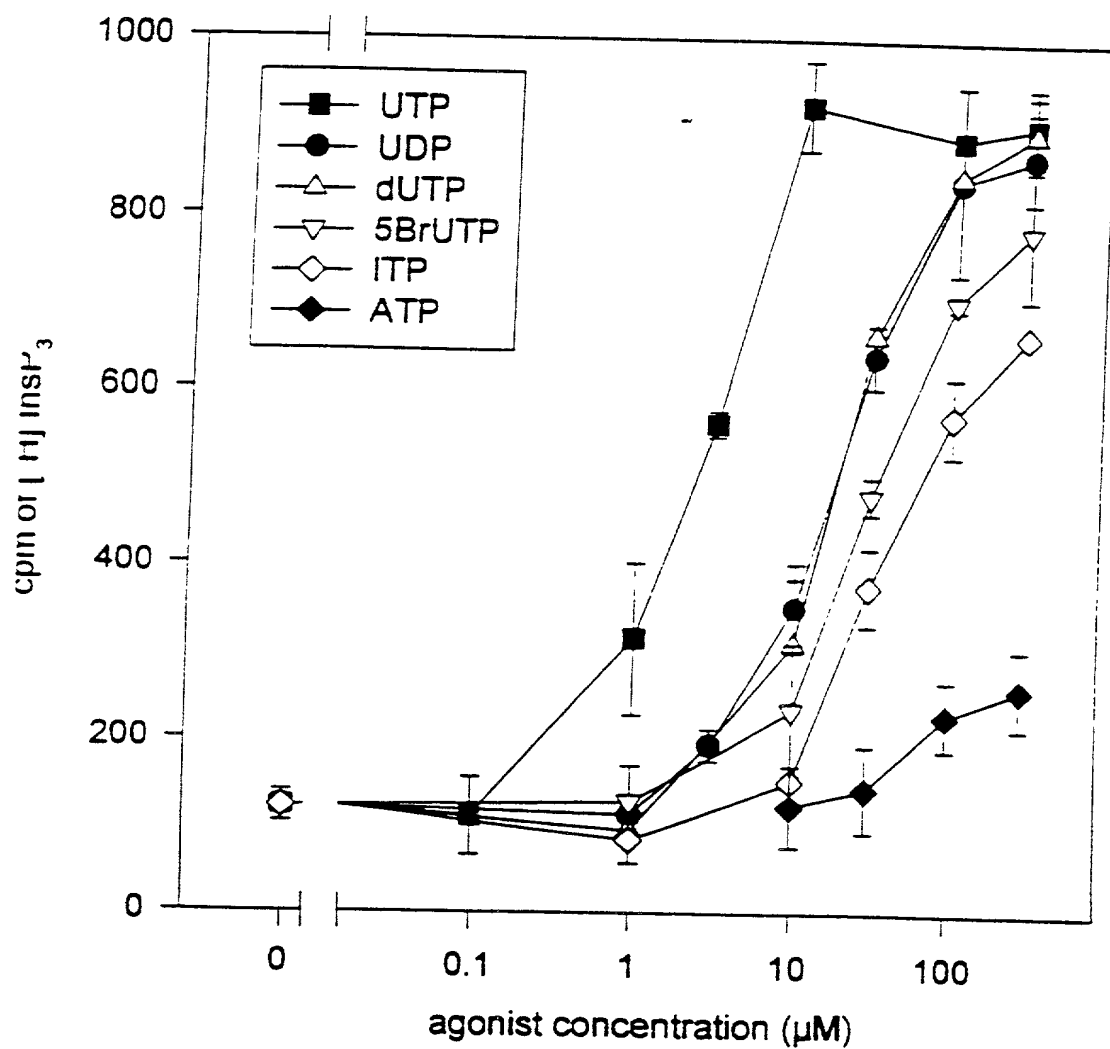


Fig.8

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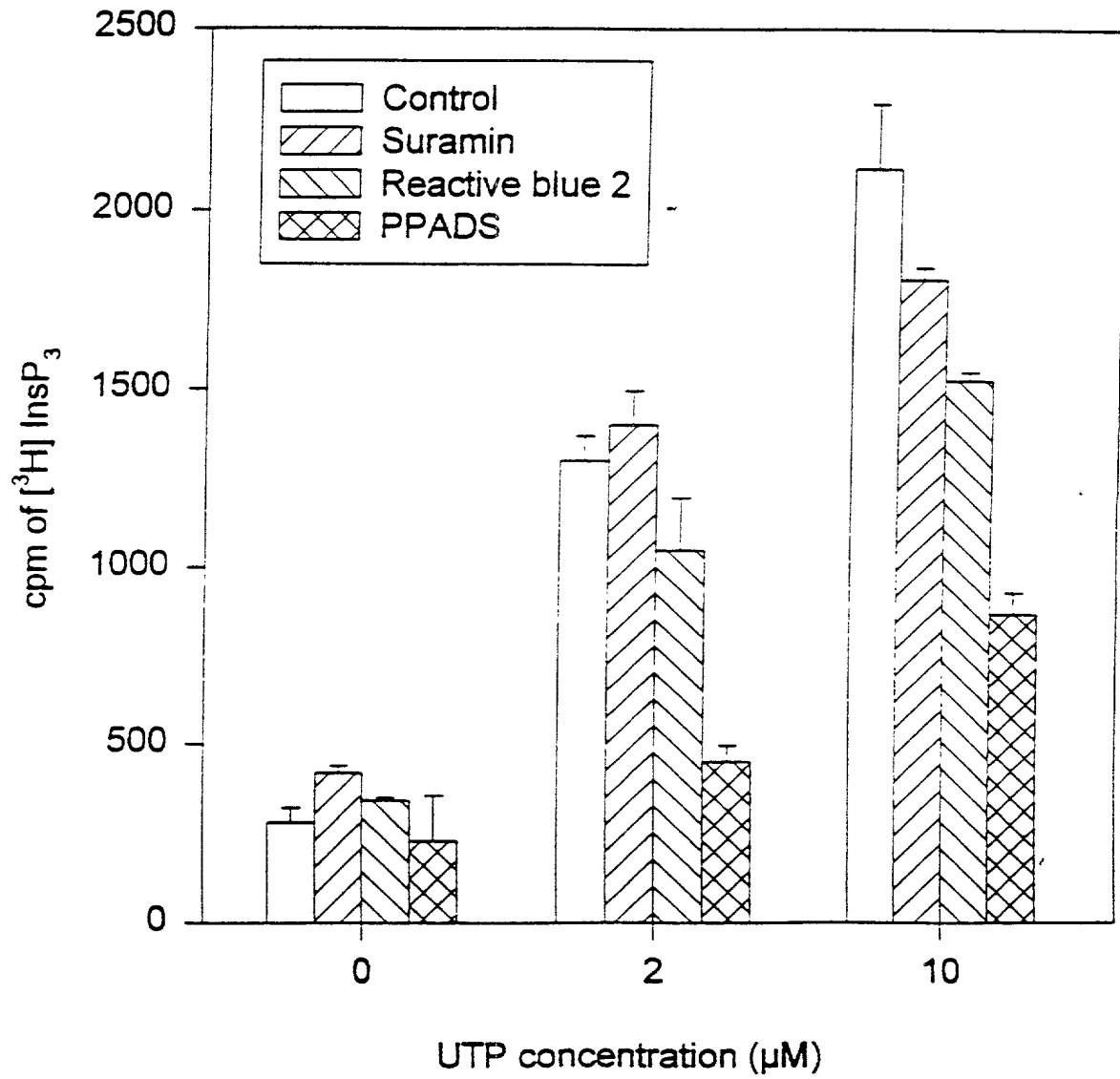


Fig. 9

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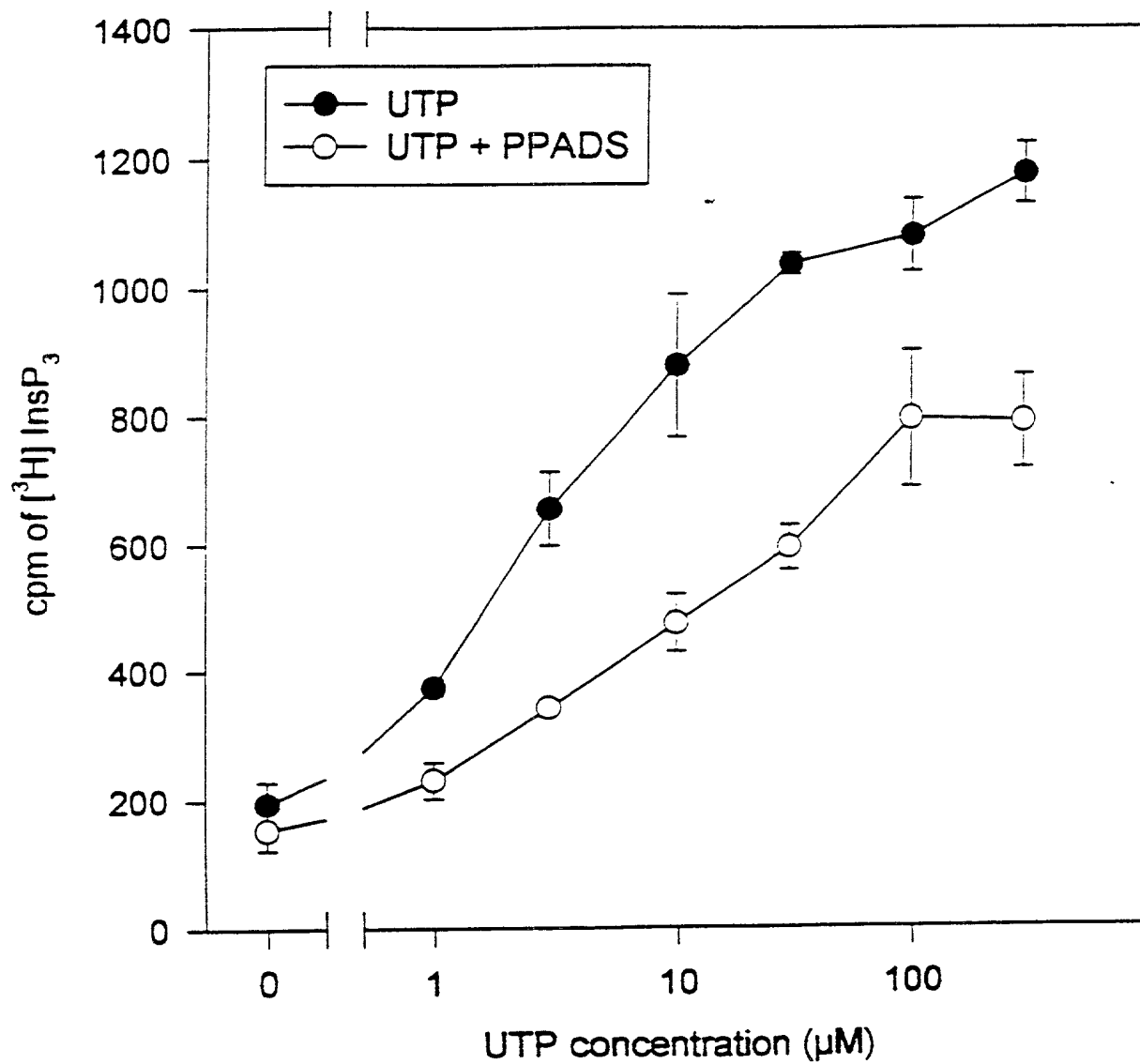


Fig. 10

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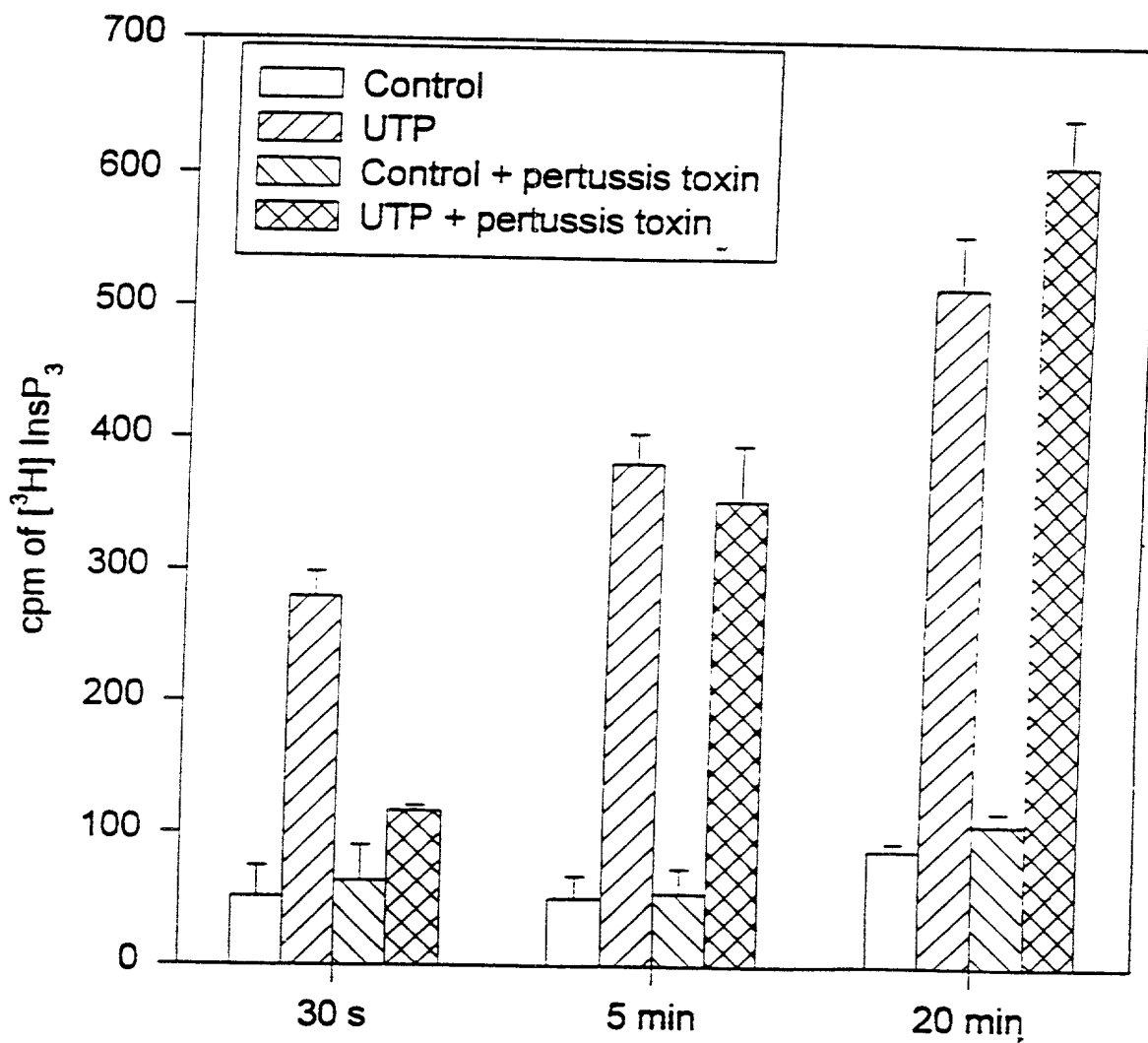


Fig. 11

DECLARATION - USA PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING SAID RECEPTOR; the specification of which was filed on **May 21, 1998** as Application Serial No. **09/077,173**.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above;

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56;

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:


PRIOR FOREIGN APPLICATION(S)

Priority
Claimed

No.: PCT/BE96/00123	Country: PCT	Date Filed: November 21, 1996	Yes
No.: 95870124.5	Country: EP	Date Filed: November 21, 1995	Yes

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature 


Date September 2, 1998

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Date September 2, 1998

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Date September 2, 1998

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